Proteinases of Legionella: Phenylalanineaminopeptidase of L. pneumophila

By SERGEI V. GUL'NIK, MARGARITA P. YUSUPOVA, GALINA I. LAVRENNOVA, IGOR S. TARTAKOVSKY, SERGEI V. PROZOROVSKY AND VALENTIN M. STEPANOV

Chemistry Department, Moscow State University, Moscow, USSR
Institute of Genetics and Selection of Industrial Microorganisms, Moscow 113545, USSR
N. F. Gamaleya Institute of Epidemiology and Microbiology, Moscow, USSR

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Phenylalanineaminopeptidase was isolated and purified from the culture filtrate of Legionella pneumophila by affinity chromatography on O-tert-butyl-L-threonyl-L-phenylalanyl-L-prolylglycyl-aminosilochrom and by gel-filtration; a 401-fold purification with a yield of 18% was achieved. The enzyme was a metalloenzyme with a molecular weight of 35000 and a pI of 5.8. It was stable at pH 7-9 and had an activity optimum in the range of pH 8-9.5 with L-phenylalanine p-nitroanilide as substrate. Enzyme activity was highest towards the latter compound, substantially lower towards L-leucine p-nitroanilide and only marginal towards other p-nitroanilides. Besides phenylalanineaminopeptidase, a metalloproteinase and a serine proteinase were also detected in L. pneumophila culture filtrate.

INTRODUCTION

Bacteria in the genus Legionella possess an unusual type of metabolism characterized by the utilization of amino acids as energy and carbon sources (George et al., 1980). There have been several reports of proteinase formation by Legionella, but no data are available on individual enzymes. Thus, L. pneumophila degraded several serum proteins including proteinase inhibitors (Müller, 1980). Enzymes in Legionella culture filtrate were reported to hydrolyse proteins (Thompson et al., 1981) as well as synthetic peptide substrates (Berdal & Fossum, 1982; Berdal et al., 1982a, b; 1983). A cell suspension of L. pneumophila hydrolysed the synthetic substrates of aminopeptidases (Müller, 1981). It was suggested that extracellular proteinases might be at least partially responsible for the pathogenicity of this bacterium (Fraser et al., 1977; Müller, 1980).

This paper deals with the isolation and biochemical characterization of phenylalanineaminopeptidase (EC 3.4.11.--) from culture filtrates of L. pneumophila, strain Philadelphia-1.

METHODS

Chemicals. p-Nitroanilides of L-phenylalanine (L-Phe), L-leucine (L-Leu), L-proline (L-Pro) and L-alanine (L-Ala) were purchased from NPO Biokhimreaktiv, Olaine, Latvian SSR; those of L-valine (L-Val), L-glutamic acid (L-Glu) and L-arginine (L-Arg) were from Serva, as was bovine serum albumin, chymotrypsinogen, myoglobin, lysozyme, cytochrome c, di-isopropyl fluorophosphate and phenylmethylsulphonyl fluoride. QAE-Sephadex A-25 and Sephadex G-75 were from Pharmacia; Acrylex P-60 was from Reanal, Hungary and ovalbumin was from Fluka. p-Nitroanilides of L-lydroglutamyl-L-alanyl-L-alanyl-L-leucine (L-Pyr-L-Ala-L-Ala-L-Leu) and of L-lydroglutamyl-L-phenylalanine (L-Pyr-L-Phe) (Oksenoit et al., 1982) were supplied by E. N. Lysogorskaya, I. Yu. Filipova and E. S. Oksenoit (Chemistry Department, Moscow State University). The affinity sorbent tetrapeptide-aminosilochrom was synthesized as described by Lyublinskaya et al. (1984).

Cultivation. L. pneumophila, strain Philadelphia-1, received from the Center for Disease Control, Atlanta, Georgia, USA, was grown in shake flasks at 200 r.p.m. at 37°C in a medium composed of (g 1-l) Difco proteose-peptone 15.0; L-cysteine 0.4; K₂HPO₄ 4.0; KH₂PO₄ 1.0; NaCl 5.0. The cells were separated from the culture medium by centrifugation for 15 min at 18000 g. The supernatant was filtered through a Millipore filter (pore diameter 0.3 μm) and stored frozen at −20°C.
Enzyme assays. Serine endopeptidase assayed at pH 8.0 using L-Pyr-L-Ala-L-Ala-L-Leu-pNA (pNA represents the p-nitroanilide group) as substrate according to the method of Lyublinskaya et al. (1977). One unit of activity was defined as the amount of enzyme releasing 1 nmol p-nitroaniline min⁻¹ at 37 °C. The molecular extinction coefficient for free p-nitroaniline was assumed to be equal to 8800 at 410 nm. Specific activity values were based on the protein content of the enzyme solution, measured as the A₄₃₀.

Aminopeptidase was assayed at pH 8.0 or 9.0 according to the method of Ivanova et al. (1977). Under the conditions of this assay the molecular extinction coefficient of p-nitroaniline was equal to 8200 at 410 nm.

Total endopeptidase activity was measured at pH 8.0 using azocasein as the substrate according to the method of Dod et al. (1978).

Isolation of phenylalanine aminopeptidase. Culture filtrate (140 ml), obtained after 20 h of cultivation, was adjusted to pH 5.6 with acetic acid; dry NaCl was then added to give a final concentration of 0.25 M. The deeply coloured solution was applied to QAE-Sephadex (3.7 x 3 cm) on a sintered glass filter. The ion exchanger had been pre-equilibrated with 0.1 M-acetate buffer, pH 5.6, containing 0.25 M-NaCl. The sorbent was washed with the same buffer and the column containing the activity towards L-Phe-pNA was collected and concentrated using an Amicon UM-2 membrane. The ultrafiltrate (22 ml) was adjusted to pH 7.5 and its NaCl concentration was increased to 1 M; it was then applied to a column of tetrapeptide-aminosilochrom (1 x 17 cm) equilibrated with 50 mM-borate buffer, pH 7.5, and 1 M NaCl. The column was then washed with the same solution (10 column vols) until the A₄₃₀ had dropped to 0.02. The active enzyme was eluted with the same buffer containing 1 M-NaCl and 25% (v/v) propion-2-ol. The eluate fractions containing aminopeptidase activity were pooled, and applied at a rate of 32 ml h⁻¹ to an Acrylex P-60 column (2.5 x 72 cm) that had been pre-equilibrated and eluted with 50 mM-borate buffer, pH 7.5, containing 0.1 M-NaCl. The active fractions (137-180 ml of the eluate) were concentrated to 3 ml using a PM-10 Amicon membrane, and then applied to a Sephadex G-75 (superfine) column (1.6 x 86 cm), which was equilibrated and eluted with 50 mM-borate buffer, pH 7.4, containing 0.1 M-NaCl. The fraction that contained pure phenylalanineaminopeptidase (75-80 ml) was kept frozen at -20 °C.

Molecular weight. This was determined by polyacrylamide gel electrophoresis (PAGE) in 0.1% (w/v) SDS (Weber & Osborn, 1969), and by gel filtration (Andrews, 1965) using a Sephadex G-75 (superfine) column equilibrated with 50 mM-borate buffer, pH 7.5, and 0.1 M-NaCl. The column was calibrated with proteins of known molecular weights: bovine serum albumin (66000), ovalbumin (45000), chymotrypsinogen (25000), and lysozyme (14300). Electrophoresis of the enzyme was done using 7.9% polyacrylamide gel at pH 8.3 and 300 V (Davis, 1964). The protein bands were stained with Coomassie brilliant blue G-250. To detect the band containing phenylalanineaminopeptidase, slices were cut from the unstained gel that corresponded to the protein bands visualized on the control gel slab. The slices were incubated with L-Phe-pNA solution at pH 8.0 and 37 °C (Kaluger et al., 1983). Isoelectrofocusing was done using a 'Multiphor' unit (LKB) in an Ampholine polyacrylamide gel plate.

Characterization of the enzyme. The pH optimum of the enzyme was determined using L-Phe-pNA as substrate at pH 5.6 (50 mM-acetate buffer), pH 6.85 (50 mM-phosphate buffer) and at pH 7.7-10.2 (50 mM-borate buffer). To assess the pH dependence of enzyme stability, 150 μl samples of phenylalanineaminopeptidase solution in 50 mM-borate buffer, pH 7.5, were added to 0.8 ml of the above mentioned buffers and the mixtures incubated for 2 h at 37 °C. Residual activity was then measured at pH 9.0.

The effect of various reagents that might inhibit or enhance enzyme activity was studied by incubating the enzyme with 1 mM-phenylmethylsulphonyl fluoride or 0.1 mM-p-hydroxymercuribenzoate for 10 min at 37 °C, and subsequently measuring activity by the usual procedure. In the control tubes the enzyme was incubated under the same conditions in the absence of these reagents. The influence of bivalent metal ions was studied by incubating the enzyme with L-Phe-pNA for 20 min at pH 8.0 (50 mM-Tris/HCl buffer) and 37 °C in the presence of 0.1, 0.01 and 0.001 mM-(CH₃COO)₂Zn, CoCl₂, CaCl₂, MnCl₂, and MgCl₂. p-Nitroaniline liberated was then determined.

Amino acid composition. The enzyme was hydrolysed in 5.7 M-HCl at 105 °C for 48 h and analysed in a Durrum D-500 amino acid analyser. No special attempts were made to assay cysteine or tryptophan or to check the determination of methionine which gave unusually high values.

RESULTS

The culture filtrate of L. pneumophila, strain Philadelphia-1, was found to contain proteolytic enzymes active against azocasein, against the p-nitroanilides of L-Pyr-L-Ala-L-Ala-L-Leu and L-Pyr-L-Phe (typical synthetic substrates for serine proteinases) and against L-Phe-pNA (a typical synthetic substrate for aminopeptidases). After 48 h of bacterial growth the following specific activities [nmol min⁻¹ (A₄₃₀ unit⁻¹)] were found: L-Phe-pNA, 3.2; L-Leu-pNA, 0.36; L-Pyr-L-Ala-L-Ala-L-Leu-pNA, 0.22; L-Pyr-L-Phe-pNA, 0.047.
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Fig. 1. Phenylalanineaminopeptidase accumulation in *L. pneumophila* culture. Culture conditions were as specified in Methods. Phenylalanineaminopeptidase activity was measured with l-Phe-pNA as substrate.

Fig. 2. pH-dependence of *L. pneumophila* phenylalanineaminopeptidase stability and activity. ▲, Activity of the enzyme against l-Phe-pNA; ■, residual activity of the enzyme after 2 h incubation at 37°C.

No activity was detected against l-Pyr-l-Ala-l-Ala-l-Phe-l-Leu-pNA and l-Pyr-l-Phe-l-Leu-pNA, substrates for papain-like thiol proteinases (Philippova *et al.*, 1984), against l-Pyr-l-Ala-l-Ala-l-Pro-pNA (specific for post-proline cleaving proteinases), or against l-Pyr-l-Tyr-l-Phe-pNA and l-Pyr-l-Phe-l-Phe-pNA. Hydrolysis of l-Pyr-l-Ala-l-Ala-l-Leu-pNA was completely inhibited by di-isopropyl fluorophosphate and by p-hydroxymercuribenzoate, which is characteristic of bacterial thiol-dependent serine proteinases (Stepanov *et al.*, 1981). Only 20% of the total activity against azocasein was inhibited by di-isopropyl fluorophosphate, whereas 80% of the activity was inhibited by Na₂EDTA, suggesting that *L. pneumophila* produces metalloproteinase(s) preferentially alongside serine (apparently thiol-dependent) proteinase. These preliminary observations on the nature of *L. pneumophila* proteinases remain to be checked by separation and thorough study of these enzymes.

*L. pneumophila* extracellular aminopeptidase was isolated in this study and, in accordance with its specificity, was named phenylalanineaminopeptidase. This enzyme appeared in the culture filtrate after 16–18 h of growth (Fig. 1); then its content rapidly increased reaching a maximum after 20–24 h. About the same time intensive pigment formation occurred. Further growth of the culture led to the fall of phenylalanineaminopeptidase activity, apparently as a result of degradation of the enzyme by other proteinases.

For the isolation of phenylalanineaminopeptidase the culture filtrate obtained after 20–24 h growth was used. The bulk of the pigment was adsorbed on QAE-Sephadex at pH 5.6, whereas phenylalanineaminopeptidase passed through the sorbent. Substantial amounts of the remaining pigment were removed by ultrafiltration. Phenylalanineaminopeptidase was further purified by affinity chromatography on tetrapeptide-aminosilochrom. This sorbent was prepared as described by Lyublinskaya *et al.* (1984) and contained tetrapeptide ligand attached to macroporous silica as shown.

\[
\text{O} \quad \text{t-But} \\
\downarrow \\
\text{l-Thr} \quad \text{l-Phe} \quad \text{l-Pro} \quad \text{Gly} \quad \text{NH} \quad \text{CH}_2\text{CH}_2\text{CH}_2\quad \text{Si} \quad \text{O} \quad \text{Si} \quad \text{O} \\
\downarrow \\
\text{O} \quad \text{O} \\
\]

This sorbent was effective in the purification of several aminopeptidases, and in the case of *L. pneumophila* phenylalanineaminopeptidase effected a 20-fold purification.
Table 1. Isolation of *L. pneumophila* phenylalanineaminopeptidase

<table>
<thead>
<tr>
<th>Step</th>
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<tr>
<td></td>
<td>Total</td>
<td>Specific</td>
<td>Yield</td>
<td>Purification</td>
<td></td>
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<tr>
<td></td>
<td>protein</td>
<td>[μmol min⁻¹]</td>
<td>(A₂₈₀ unit⁻¹)</td>
<td>(fold)</td>
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<td>Culture filtrate</td>
<td>1115</td>
<td>1.69 × 10⁻²</td>
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<tr>
<td>QAE-Sephadex A-25</td>
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<td>2.71 × 10⁻²</td>
<td>114</td>
<td>1.6</td>
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<tr>
<td>Ultrafiltration</td>
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<td>4.8 × 10⁻²</td>
<td>83</td>
<td>2.9</td>
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<tr>
<td>Affinity chromatography on tetrapeptide</td>
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<td>58</td>
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<td>aminosilochrom</td>
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Table 2. Amino acid composition (residues per molecule) of phenylalanineaminopeptidase

<table>
<thead>
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<th>Amino acid</th>
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<td>Asp</td>
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<td>Ile</td>
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<tr>
<td>Thr</td>
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<td>Leu</td>
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<td>Phe</td>
<td>10</td>
</tr>
<tr>
<td>Pro</td>
<td>22</td>
<td>His</td>
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<td>Gly</td>
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</tr>
<tr>
<td>Ala</td>
<td>39</td>
<td>Arg</td>
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</tr>
<tr>
<td>Val</td>
<td>19</td>
<td>Trp</td>
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</tr>
<tr>
<td>Met</td>
<td>11</td>
<td>Cys</td>
<td>ND</td>
</tr>
<tr>
<td>Total</td>
<td>340</td>
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ND, Not determined.

The enzyme was sorbed on tetrapeptide-aminosilochrom at pH 7.5 in the presence of 1 M NaCl to reduce nonspecific binding of extraneous proteins. The enzyme was desorbed by 25% (v/v) propan-2-ol, the latter apparently suppressing hydrophobic contacts between the enzyme and the ligand. As the final steps in the purification two gel filtration procedures – through Acrylex P-60 and Sephadex G-75 – were used (Table 1).

The homogeneity of the isolated phenylalanineaminopeptidase was confirmed by the presence of one protein band, *R*ₚ 0.6 (relative to the marker dye mobility), possessing activity against L-Phe-pNA and L-Leu-pNA, after PAGE at pH 8.3. SDS-PAGE also revealed only one protein band corresponding to a molecular weight of 35000. The same value was found for the native enzyme by gel filtration through Sephadex G-75 suggesting that the enzyme consists of a single polypeptide chain. Isoelectrofocusing in polyacrylamide gel also revealed a single protein band of pI 5.8.

Phenylalanineaminopeptidase was characterized by a rather high content of dicarboxylic amino acids and their amides – 83 residues out of 340 – whereas only 25 basic residues were found (Table 2). This explained the low isoelectric point of the enzyme. The enzyme was stable within the pH range 7–9; the optimum pH for its activity was in the range pH 8.0–9.5 (Fig. 2). The enzyme was especially active against L-Phe-pNA with considerably less activity towards L-Leu-pNA (17% of the specific activity shown for L-Phe-pNA). Its action on the p-nitroanilides of L-Val, L-Glu, L-Ala, L-Arg and L-Pro was negligible. However, the influence of secondary specificity broadened this specificity pattern if peptides rather than amino acid p-nitroanilides were used as the substrates. Indeed, rather extensive cleavage of leucine, proline, valine and isoleucine residues was observed when the following peptides were hydrolysed by *L. pneumophila* phenylalanineaminopeptidase for 18 h at pH 9.0 and 37 °C (residues removed totally are given in bold type): Leu-Gly-Gly, Pro-Leu-Gly-NH₂, Val-Val; Ile-Ser-Asp-Thr-Asn-Gln; Ile-Val-Asp-Thr-Gly-Thr-Ser-Leu. The glycyl peptides Gly-Asn, Gly-Ala, Gly-Met, Gly-Phe, Gly-Leu, Gly-
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Trp, Gly-Gly, Gly-Gly-NH₂ and Gly-Gly-Gly were completely resistant to the enzyme. Hence, the specificity of phenylalanineaminopeptidase is considerably broader than might be concluded from the hydrolysis of a range of amino acid p-nitroanilides.

The enzyme was completely inhibited by 1 mM-Na₂EDTA, 4 mM-cysteine, 1 mM-mercaptoethanol and 1 mM-dithiothreitol. This indicated the specific role of a metal ion in enzyme activity. Bivalent metal ions – Zn²⁺, Ca²⁺, Mg²⁺, Mn²⁺, Co²⁺ – did not influence enzyme activity at concentrations up to 10 μM. Neither 1 M-NaCl nor 1 M-LiCl influenced enzyme activity whereas 1 M-KCl decreased the activity by about 50%. p-Hydroxymercuribenzoate also inhibited the enzyme by about 50% but phenylmethylsulphonyl fluoride had no effect.

DISCUSSION

L. pneumophila forms substantial amounts of phenylalanineaminopeptidase. The role of this enzyme is apparently the liberation of free amino acids, especially hydrophobic ones, from the peptides present in the growth medium. Under physiological conditions the enzyme might act on the host intracellular peptides or the products of the host proteins digested by Legionella secretory proteinases, including the metalloproteinase. A more precise description of the way this bacterium produces free amino acids requires more extensive characterization of its proteinases.

Unfortunately, the paucity of the data on microbial aminopeptidases as well as the vagueness of Legionella taxonomy handicaps any comparison of L. pneumophila phenylalanineaminopeptidase with other microbial aminopeptidases. Several aminopeptidases secreted by bacteria are metalloenzymes of relatively low molecular weight and are devoid of quartenary structure. Thus, the molecular weight of Aeromonas proteolytica aminopeptidase is 29 500 (Prescott et al., 1971), that of Vibrio SA 1 is 21 000 (Wiersma et al., 1978) and that of Bacillus licheniformis is 37 500 (Rodriguez-Abshi & Prescott, 1978). The molecular weight of the L. pneumophila enzyme, at 35 000, is obviously of the same magnitude. Metal ions are apparently involved in L. pneumophila aminopeptidase activity. Similarly, the aminopeptidases from A. proteolytica (Prescott et al., 1971), Vibrio SA 1 (Wiersma et al., 1978) and Alteromonas B-207 (Merkef et al., 1981) also contain metal ions in their active sites. The pH-optimum of the L. pneumophila aminopeptidase is similar to those of the bacterial aminopeptidases mentioned above.

To our knowledge, the remarkable preference for L-Phe-pNA shown by the L. pneumophila enzyme is not matched by other bacterial proteinases, which as a rule prefer leucine as the N-terminal amino acid (Wagner et al., 1972; Wiersma et al., 1978; Rodriguez-Abshi & Prescott, 1978; Merkef et al., 1981). Aminopeptidases that preferentially cleave N-terminal phenylalanine have however, been found in several plants (Kolehmainen & Mikola, 1971; Vodkin & Scandalious, 1980; Waters & Dalling, 1984; Ninomiya et al., 1981). These aminopeptidases are apparently not metalloenzymes and are thus distinguished from L. pneumophila phenylalanineaminopeptidase.

Phenylalanineaminopeptidase isolated from Trichoderma viride (Vaganova et al., 1983) also shows a strong preference for L-Phe-pNA. Hence, several aminopeptidases of different origin share the same pattern of substrate specificity which is characterized by a preference for L-Phe-pNA. L. pneumophila phenylalanineaminopeptidase belongs to this type.

The authors are indebted to Dr E. A. Timokhina for amino acid analyses.

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